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Modulation of the gap junction protein Connexin36 in neurons in a mouse model of transient focal cerebral ischemia

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Summary

Intercellular communication is achieved at specialized regions of the plasma membrane by gap junctions. Gap junctions are transmembrane channels allowing direct contacts between the cytoplasms of neighboring cells. Each cell participates with one hemichannel, or connexon, made of six protein subunits named connexins. Thanks to these junctions, cells potentially share a pool of small molecules and metabolites, such as nucleotides, amino acids and second messengers.

In an ischemic (i.e. non-perfused) territory of the brain, irreversible damage progresses over time from the centre of the most severe flow reduction to the periphery with less disturbed perfusion. Functionally impaired tissue can survive and recover if sufficient reperfusion is re-established within a limited time period, which depends on various factors and mechanisms modulating the signaling pathways leading to cell death.

Observations were made indicating the presence of electrical coupling between neurons which resist better to an ischemic insult. This electrical coupling is likely to be mediated by Connexin36 (Cx36), a neuron specific connexin isoform. It was demonstrated in the past that global ischemia induces a selective upregulation of Cx36 expression in regions with neurons that survive the insult whereas others undergo apoptosis and die. These observations raise the possibility that the neuronal gap junction Cx36 might play a role in the destiny of neurons after cerebral ischemia.

The aim of this work was to characterize the regulation of Connexin36 in a mouse model of transient focal cerebral ischemia by immunofluorescence and Western blot analysis. Our immunofluorescence results suggest a specific increase in Cx36 in the penumbral region of the ischemic hemisphere.

Key words: Intercellular communication, Connexin, Cerebral ischemia

Table of contents

1. INTRODUCTION

1.1 Connexin, connexon, gap junction

Gap junctions are specialized membrane regions composed of aggregates of transmembrane channels that directly connect the cytoplasm of adjacent coupled cells. These intercellular channels are present in almost all cell types of vertebrate organisms. The passage of ions and small molecules with a molecular weight smaller than 1,2 kDa through the gap junction-channels results in metabolic and electrical coupling of cells (Condorelli, Belluardo et al. 2000). As a result, gap-junctions synchronize activity of coupled cells and are thought to play an important role in intercellular signalling in brain development, morphogenesis and pattern formation (Goodenough and Paul 2009). The gap junction comprises two apposed hexameric structures called connexons (or hemichannels), one contributed by each cell. These connexons eventually contact each other to bridge a gap of approximately 3nm between the two cell membranes. Each connexon is composed of six connexin subunits surrounding a central pore (Figure1).

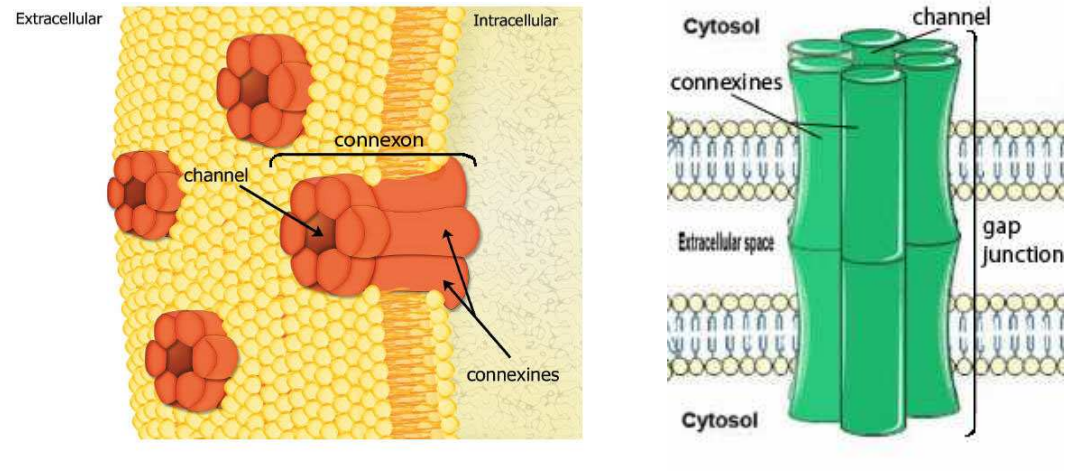

Figure 1) Connexin, connexon, gap junction
(adapted from Allagnat, Thesis, 2005; Alonso, Thesis 2011)

Connexins are encoded by a gene family of at least 20 structurally related members in mammals. Although connexins share sequence similarity and a common membrane topology,

they assemble and form channels that differ in gating and permeability properties as well as temporal and spatial patterns of expression (Oguro, Jover et al. 2001).

In the nervous system, gap junction-mediated communication is the most common form of electronic coupling between neurons. Interneuronal gap junctions provide the structural basis for the so-called “electrical synapses”, where multiple gap junctions assemble to form an array (“plaque”) and couple mechanically and electrically two adjacent neurons thus providing a conductive link for exchanging signals (Condorelli, Belluardo et al. 2000).

1.2. Different cell types in the brain

The brain is composed of neurons and a special connective tissue called glial tissue (Figure 2). Although neurons vary in structure, functional interconnections and biochemical properties, they share some fundamental aspects: a neuron is an electrically excitable cell transmitting information by electrical and chemical signalling. Neurons transmit information through cytoplasmic prolongations, called axons, building neuronal circuits leading to a network which gives rise to the possibility of integrating, processing and transmitting information. Neurons are, compared to other cell types, relatively unable to regenerate. This means that focal neuronal loss can result in permanent symptomatic neurologic deficits ((Kumar and Robbins 2007).

Glial cells (astrocytes, oligodendrocytes and microglia) provide a support function for neurons and their cellular processes. They also have a primary role in repair, fluid balance and energy metabolism.

Astrocytes (so called because of their stellate morphology) have processes that are directed toward capillaries and neurons. Important normal functions include structural support, contributions to the blood-brain barrier and action as metabolic buffers or detoxifiers. They are the principal cells responsible for repair and scar formation in the brain. ((Kumar and Robbins 2007). Astrocytes contain the intermediate filament glial fibrillary acidic protein (GFAP) which can be used as a cell-type specific marker for differentiated astrocytes. When

there is a brain lesion (such as after an ischemic insult), astrocytes become reactive, creating a physical barrier between healthy and damaged tissue which can be identified by the study of anti-GFAP immunofluorescence (Benakis, Bonny et al. 2010).

Oligodendrocytes are the cells producing the myelin sheats which surround neuronal axons, providing acceleration of the conduction process of information. Microglia are mobile, acting as cells of the immune system in the brain, they phagocyte damaged tissue, microorganisms, as well as antibody-antigen complexes. (Waxman 2010).

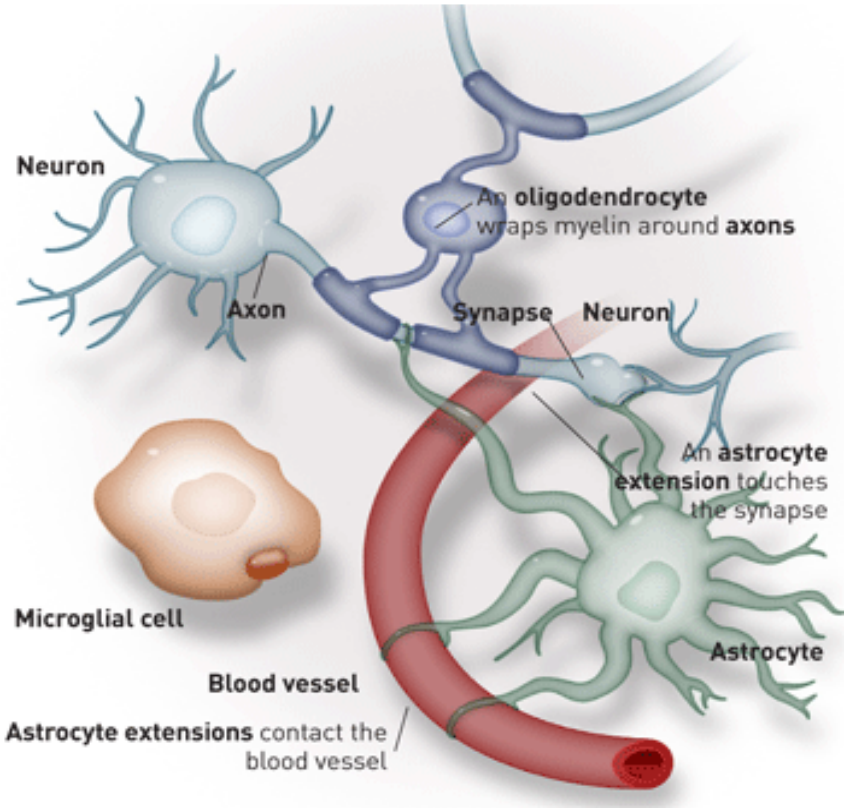

Figure 2) Different cell types in the brain
http://www.nature.com/nature/journal/v457/n7230/fig_tab/457675a_F1.html

1.3. Connexins in the brain

Three gap junction-proteins are expressed abundantly in mammalian brain (but with differing cellular specificity): Connexin 32 (Cx32), Connexin 36 (Cx36) and Connexin 43 (Cx43). Whereas Cx43 is the most abundant connexin expressed by astrocytes, Cx32 is expressed predominantly in oligodendrocytes. Cx36 protein expression is neuron specific (Condorelli, Trovato-Salinaro et al. 2003; Sohl, Maxeiner et al. 2005). Characterizing temporal, spatial and

cellular differences in connexin expression is crucial to better understand their role in the brain in normal and pathophysiological conditions.

1.4. Cerebral ischemia

Cerebral ischemia (i.e. non-perfusion of the brain) is most often caused by a cerebrovascular accident which denotes any abnormality of the brain caused by a pathologic process involving blood vessels. The three basic mechanisms are thrombotic occlusion of vessels, embolic occlusion of vessels and vascular rupture (Figure 3). The first two share many characteristics, because their effect on the brain is the same: Loss of oxygen and metabolic substrates finally resulting in brain infarction. Thrombosis and embolism cause ischemic injury or infarction of specific regions of the brain, depending on the vessel involved. A similar pattern of injury occurs diffusely when there is complete loss of brain perfusion. Rupture of vessels leads to direct tissue damage as well as secondary ischemic injury. A “stroke” is the clinical designation that applies to all these conditions, particularly when symptoms begin acutely. The stroke or cerebrovascular accident remains a major health problem and is a leading cause of death and disability (Kumar and Robbins 2007).

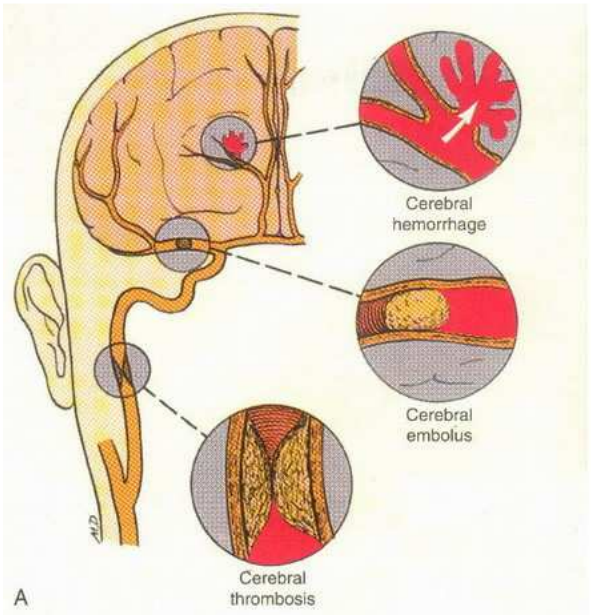

Figure 3) Mechanisms leading to a cerebrovascular accident
http://www.lookfordiagnosis.com/images.php?term=Cerebrovascular+Accident&lang=1&from=8

Cerebral ischemia induces neuronal damage in the territory of the non-perfused artery. The rapid decrease of oxygen and glucose in the ischemic region can cause profound cellular dysregulations within minutes. The fundamental biochemical abnormality in hypoxic neurons that leads to cell injury is reduced intracellular generation of adenosinetriphosphate (ATP), as a consequence of absent supply of O_2. Loss of ATP leads to the failure of many energy-dependent cellular systems, including ion pumps (leading to swelling and influx of Ca^{++}), depletion of glycogen stores (with accumulation of lactic acid, thus lowering the intracellular pH) and reduction of protein synthesis (Kumar and Robbins 2007).

In early stages or mild forms of injury, the functional and morphologic cell changes are reversible if the damaging stimulus is removed. With continuing damage, the injury becomes irreversible, at which the cell cannot recover and dies.

In an ischemic territory of the brain, irreversible damage progresses over time from the centre of the most severe flow reduction to the periphery with less disturbed perfusion. The zone between damaged and the functional brain tissue is referred to as the penumbra. The concept of penumbra during focal cerebral ischemia refers to the regions of brain tissue where blood flow is sufficiently reduced to cause hypoxia arresting cell function, but not so complete as to cause irreversible failure of energy metabolism resulting in cell death. As tissue tolerance to ischemic damage is dependent on residual blood flow and duration of flow disturbance, ischemic penumbra is a dynamic process. It exists for a short time even in the center of an ischemic region, from where cell death propagates to the neighboring tissue over time (Paciaroni, Caso et al. 2009).

There are two types of cell death, necrosis and apoptosis, which differ in their morphology, mechanisms and roles in cerebral ischemia. The term necrosis refers to a series of changes that accompany cell death in lethally injured cells which are unable to maintain membrane integrity. When damage to membranes is severe, enzymes leak out of lysosomes, enter the cytoplasm, and digest the cell, resulting in necrosis. Cellular contents also leak out and elicit a

host reaction (inflammation). When a cell is deprived of growth factors or the cell's DNA or proteins are damaged beyond repair, the cell kills itself by another type of death, called apoptosis. Apoptosis is an active, energy-dependent pathway of cell death that is induced by a tightly regulated suicide program in which cells destined to die activate enzymes degrading DNA as well as nuclear and cytoplasmic proteins. The fundamental event in apoptosis is the activation (=cleavage of zymogens into active forms) of enzymes called caspases (so named because they are cysteine proteases that cleave proteins after aspartic residues). The activation of caspases depends on a finely tuned balance between pro- and anti-apoptotic signals and intracellular proteins (Kumar and Robbins 2007).

In a cerebrovascular accident, the first wave of neuronal death is mainly by necrosis in the immediate vicinity of the center of the ischemic region, while apoptosis is the main form of cell death at later time points in the progressing penumbra (Nakase, Fushiki et al. 2003; Talhouk, Zeinieh et al. 2008).

The penumbra concept furthermore suggests that functionally impaired tissue can survive and recover if sufficient reperfusion is re-established within a limited time period, which depends on various factors and mechanisms modulating the signaling pathways leading to apoptosis. One of the challenges in understanding the dynamics of cell survival during reperfusion of the penumbral region is that it relies on complex interplay between the different cell-types present in the central nervous system, especially since they exhibit different resistances to an ischemic insult such as a cerebrovascular accident (Kumar and Robbins 2007). At any given survival time, one region may include subpopulations of dead, damaged and regenerating cells that influence each other in ways that still need to be elucidated (Talhouk, Zeinieh et al. 2008).

As neurons have a high demand on energy, they are especially sensitive to O_2 and glucose deprivation occurring during ischemia. Astrocytes, which are more resistant than neurons, are initially activated in the lesion, produce various cytokines, interact with inflammatory cells and accumulate glycogen for energy supply under ischemic conditions (Mehta, Manhas et al.

2007; Tuttolomondo, Di Raimondo et al. 2008; Xia, Han et al. 2010). They maintain the extracellular glutamate concentration, thereby reducing metabolic stress to neurons associated with activity and reduce cytotoxic reactive oxygen species accumulation (Kunz, Park et al. 2007). Therefore, astrocytes are considered to protect neurons from ischemic insults.

1.5. Gap junctions in cerebral ischemia

The intercellular communication and regulatory mechanisms taking place in an ischemic brain region described above are thought to be, at least partially, mediated via gap junctional intercellular signalling. In vitro blocking of gap junctions in neuron-astrocyte co-cultures leads to a decrease of these protective mechanisms (Ozog, Siushansian et al. 2002). On the other hand, there is evidence supporting a role for interastrocytic gap junctions in the spread of secondary injury associated with focal ischemia: gap junctions between dying glial cells such as astrocytes can kill resistant neighboring glial cells via glial “fratricide” (bystander death) and thereby propagate and spatially amplify injury (Lin, Weigel et al. 1998). Under ischemic conditions, the pro- or antiapoptotic role of astrocytic gap junctions is still controversial in literature (Nakase and Naus 2004; Talhouk, Zeinieh et al. 2008).

In an animal model, transient cerebral ischemia induces selective, delayed neuronal death. Some neurons in the brain are resistant to ischemic insults that kill others and survive (Oguro, Jover et al. 2001). The molecular mechanisms underlying the cell-specific pattern of ischemia-induced neuronal death are not well understood.

There is morphological evidence indicating the presence of electrical coupling between neurons which resist better to an ischemic insult (Oguro, Jover et al. 2001). Observations were made indicating that this electrical coupling is likely to be mediated by Cx36, which is highly expressed in these neurons (Condorelli, Belluardo et al. 2000). Transient global ischemia induces a selective upregulation of Cx36 expression in neurons that survive the insult whereas others undergo apoptosis and die (Oguro, Jover et al. 2001). These observations raise the

possibility that Cx36 gap junctions might play a role in the destiny of neurons after transient ischemia.

Gap junctions linking these neurons are thought to synchronize and reinforce inhibitory synaptic transmission supporting a role for gap junctions in promotion of resistance and survival of selected neuronal populations to ischemia-induced damage. The inhibitory signal is thought to reduce vulnerability because of reduced metabolic stress associated with less neuronal activity (Oguro, Jover et al. 2001).

The present work was undertaken to examine the expression of Cx36 in neurons in the penumbral region of a focal ischemic lesion in the mouse brain. The lesion was induced by selective transient middle cerebral artery occlusion as stroke model.

2. MATERIAL AND METHODS

2.1. Animals

Adult wild type ICR mice (20-35g), 4-5 weeks old were used by the group of Prof. Lorenz Hirt, Laboratory of Neurology, CHUV, Lausanne.

2.2. Transient middle cerebral artery occlusion (MCAo)

The mouse model of the MCAo was performed by Dr. Wilfredo Puentes from the group of Prof. Lorenz Hirt, Laboratory of Neurology, CHUV, Lausanne as previously described (Benakis, Bonny et al. 2010). Mice where anesthetized and maintained with 1-3% isoflurane in 70% N_2O and 30% O_2 using a face mask. Body temperature was maintained at 37± 0,5° C throughout surgery (FHC Inc., Bowdoinham, ME, US). Regional cerebral blood flow was continuously recorded by laser Doppler flowmetry (LDF, Periflux 5000, Perimed, Sweden) during a period covering the induction of ischemia until 10min after the end of ischemia. Transient focal cerebral ischemia was induced by the following steps: ligature of left proximal arteria carotis communis near the aortic arch and ligature of left proximal arteria carotis externa, temporary clip on left distal arteria carotis interna and incision of the now non-perfused left arteria carotis communis to introduce a silicone coated nylon monofilament (diameter: 0,17mm, Docol Co., Redlands, CA, US) through the left arteria carotis communis. Ligature and fixation of the nylon monofilament on proximal left arteria carotis interna. Withdrawal of monofilament after 30min to allow reperfusion. Mice were given 0,025mg/kg of buprenorphine subcutaneously for post-surgery analgesia and were housed in an incubator at 31° C during 24h for recovery.

2.3. Tissue preparation

Mice were profoundly anesthetized with intraperitoneal xylazine injection, killed by cervical dislocation and transcardially perfused with phosphate-buffered saline (PBS), pH 7,4 injected into the left ventriculum. Simultaneously, the vena cava inferior was dissected allowing the blood and PBS to exit the vascular system. Mice where then decapitated and the brain

extirpated. Isolated brains were positioned slightly above liquid nitrogen in a recipient so that it can be frozen by the vapour of the nitrogen. Sections of the frozen specimen were cut with a cryostat (Cryostat CM3050 S, Leica Microsystems, Wetzlar, Germany) at -10° C and a chamber temperature of -15° C. Serial coronal 10µm thick, 700µm distant frozen sections were cut for immunolabelling. 25µm and 50µm thick coronal slices between the 10µm slices were cut for cresyl violet staining and Western blot analysis respectively. Brain sections were mounted on glass plates (SuperFrost Ultra Plus, Menzel, Braunschweig, Germany) and kept at -80° C.

2.4. Cresyl violet staining

For a macroscopic analysis, neuronal damage was assessed by histological examination of the damaged as well as the control tissue of brain sections at the level of the lesion, 25µm thick slices were stained in cresyl violet by the group of Prof. Lorenz Hirt, Laboratory of Neurology, CHUV, Lausanne.

2.5. Immunofluorescence analysis

For qualitative evaluation as well as anatomical localization of apoptosis in the ischemic region, we used rabbit anti-cleaved caspase 3 antibodies (Cell Signaling Technology, Danvers, MA, US) on 10µm thick coronal sections of the brain. For the cleaved caspase 3 marking, the cryoconservated sections were rehydrated in phosphate-buffered saline (PBS), fixed during 10min with 4% Paraformaldehyd in PBS and permeabilized in phosphate-buffered saline-bovine serum antigen (PBS-BSA) 1,5% Triton 0,1% during 1 hour at room temperature. Then, sections were incubated overnight at 4° C with anti-cleaved caspase 3 antibodies (1:500) in PBS-BSA 1%. The primary antibodies were visualized using goat anti-rabbit Alexa Fluor 488 (1:1000, Invitrogen, Carlsbad, CA, US) in PBS-BSA 1%.

For the anti-Cx36, anti-Cx43 and anti-GFAP immunofluorescence studies, 10µm thick coronal sections of the brain were rehydrated in PBS followed by permeabilization in PBS-BSA 1,5% Triton 0,1% during 1 hour at room temperature. Then, sections were incubated

overnight at 4° C alone or in combinations of the following primary antibodies diluted in PBS-BSA 1%: mouse anti-GFAP (1:250, Millipore, Billerica, MA, US), rabbit anti-Cx43 (1:1000; Cell Signalling Technology, Danvers, MA, US), rabbit anti-Cx36 (1:50; Invitrogen, Carlsbad, CA, US).

Antigens were visualized with the appropriate fluorochrome-conjugated secondary antibodies diluted in PBS-BSA 1%: goat anti-mouse Alexa Fluor 594 (1:400, Invitrogen, Carlsbad, CA, US), goat anti-rabbit Alexa Fluor 488 (1:1000, Invitrogen, Carlsbad, CA, US) respectively. Sections were counterstained with DAPI and mounted in Mounting Medium (Vectashield, Vector Laboratories, Burlingame, CA, US) diluted 1:3 in 50% glycerol. Images were acquired with a Leica microscope (Leica Microsystems, Wetzlar, Germany).

2.7. Western blot analysis

For quantification of protein abundance in the lesion and the surrounding ischemic penumbra, 50µm coronal slices were cut on a cryostat (Cryostat CM3050 S, Leica Microsystems, Wetzlar, Germany). To minimize "dilution" of the ischemic tissue of interest, only slices with a macroscopically visible lesion were used for Western blot analysis. The slices were cut in half along the fissura longitudinalis cerebri and around the macroscopically visible lesion to reduce the amount of non ischemic tissue in the sample used for analysis of the ischemic region. Tissue samples were homogenized by sonication in sodium dodecylsulfate (SDS) lysis buffer solution (62.5mM Tris-EDTA, pH 6.8, 5% SDS). Protein concentrations of the samples were measured using the DC protein assay (Bio-Rad, Hercules, CA, US). Samples were diluted in SDS lysis buffer to achieve the same final protein concentration, proteins were denaturated by loading buffer and 5min heating in a thermoblock at 98° C.

Samples containing the same final protein amount were loaded onto 12% polyacrylamide gels and subjected to gel electrophoresis.

Protein bands were transferred to immobilon PVDF membranes (Millipore, Billerica, MA, US). Membranes were blocked during 1h at room temperature in blocking buffer (TBS-

Tween 0.1%-skim milk 5%). Then, membranes were incubated with a primary antibody (anti-Cx36 1:100, anti-Cx43 1:100) in TBST-BSA 5% overnight in a 4° C cold-chamber. After 3x10min TBST washes, the membranes were incubated in goat anti-rabbit antibodies coupled to the horseradish peroxidase (HRP) diluted in blocking buffer (1:10’000, Millipore, Billerica, MA, US) during 1h at room temperature. After reaction and 3x10min consecutive TBST washes, membranes were treated with enhanced chemiluminescence reagents (HRP Immobilon substrate, Millipore, Billerica, MA, US).) and exposed to Kodak films (Kodak, Rochester, NY, US).

3. RESULTS

3.1. Transient middle cerebral artery occlusion induces a visible lesion on cresyl violet stained mouse brain slices

To examine ischemia-induced alterations in connexin expression in the mouse brain, mice were subjected to transient focal cerebral ischemia by selective transient middle cerebral artery occlusion (MCAo) for 30 min and housed during 24h before sacrifice. To assess qualitatively brain damage in the territory of the occluded artery after MCAo, brain sections were subjected to macroscopic histological analysis (Figure 4). At 24h after a 30min MCAo, almost all mice showed a histologically visible lesion in cresyl violet staining which presented as a region with less intense staining in the left hemisphere (Figure 4B and C). The left hemisphere, which was subjected to 30min of MCAo, showed either one of the following two lesion patterns: A smaller, striatal lesion which affects only the striatum and seems to be limited by the corpus callosum (Figure 4B) or, a bigger, corticostriatal lesion, extending from the striatum into the left cortex (Figure 4C).

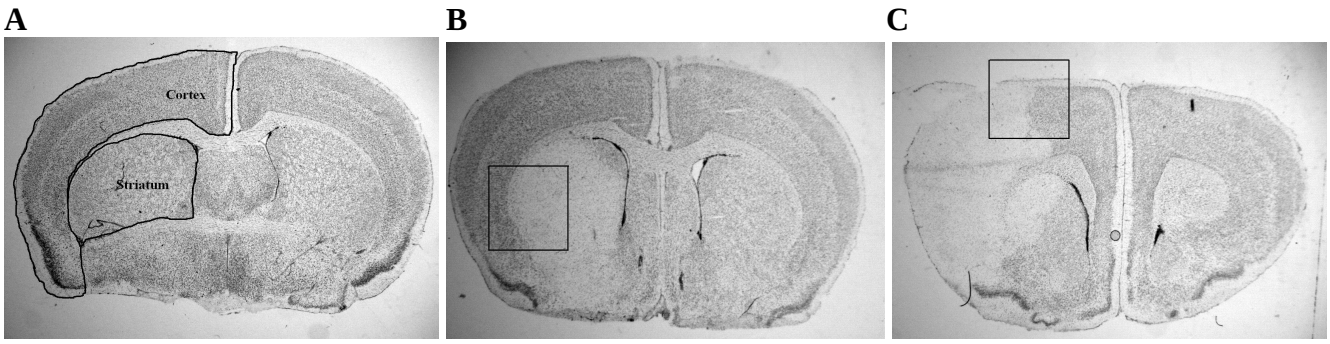

Figure 4) Cresyl Violet staining on mice brain 24h after 30min MCAo: A: sham, B: striatal lesion, C: corticostriatal lesion. Squares indicate the determined regions of interest.

3.2. Cleaved caspase 3 immunofluorescence shows a visible frontier between labelling-positive and negative cells in the hemisphere with focal cerebral ischemia

For a more detailed evaluation of the damaged hemisphere, we used cleaved caspase 3 immunofluorescence which allowed us to confirm or exclude the presence of apoptosis in the ischemic and the adjacent normal brain parenchyma (Figure 5). We found that the localisation

of cleaved caspase 3 labelling was identical to the lesion observed on the cresyl violet stained slices. We concluded that the external limit of the area containing cleaved caspase 3 positive cells should be the region of interest to be observed with the consecutive immunofluorescence methods.

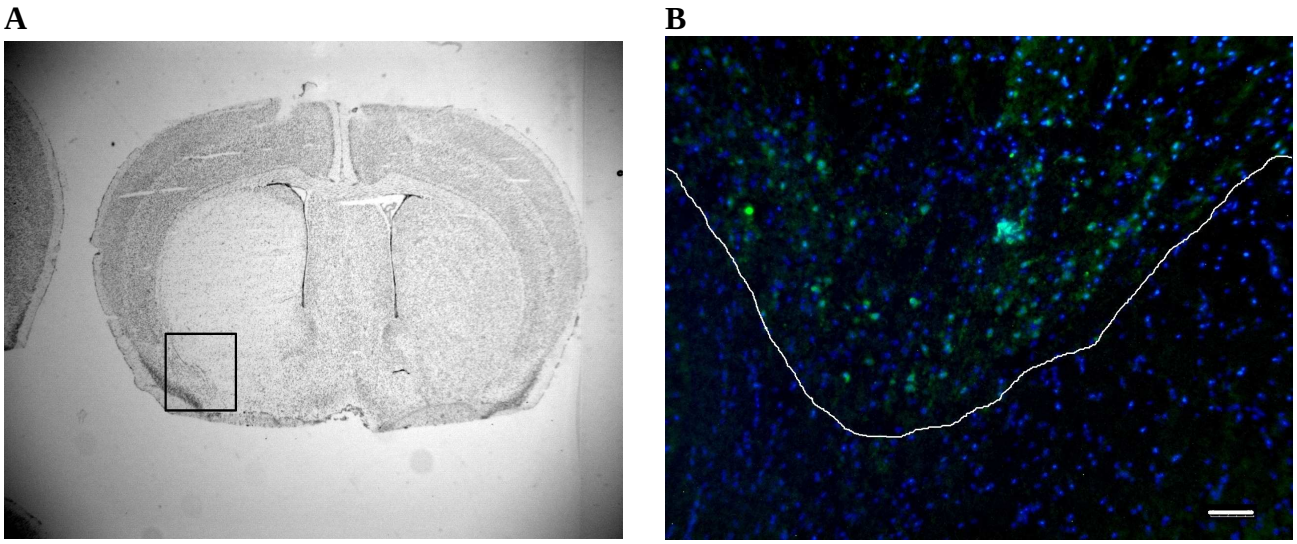

Figure 5) Anti-cleaved caspase 3 immunofluorescence: A: Cresyl violet staining, square indicates the region on the border of the lesion showed in B. B: a limit is traceable between the cleaved caspase 3 labelling-positive and the adjacent non-apoptotic, only DAPI stained nuclei. Scale bar=50µm

3.3. Focal cerebral ischemia modulates Connexin36 levels

To examine ischemia-induced alterations in Cx36 expression, consecutive slices from the same mice brains used for cresyl violet staining and anti-cleaved caspase 3 were used. Immunofluorescence studies were performed using specific antibodies against Cx36 in order to qualitatively compare the membraneous expression of Cx36 of neurons on the left hemisphere subjected to 30min MCAo in the ischemic region and the controlateral control hemisphere. We studied Cx36 in the lesion region defined by the cresyl violet staining and the anti-cleaved caspase 3 labelling, which is the region containing potentially viable but not functional brain tissue and where recovery as well as degeneration are equally possible(Figure 6). 30min of focal cerebral ischemia markedly increases the specific punctate Cx36 immunolabelling in the ischemic region compared to the same region on the controlateral, non-ischemic hemisphere.

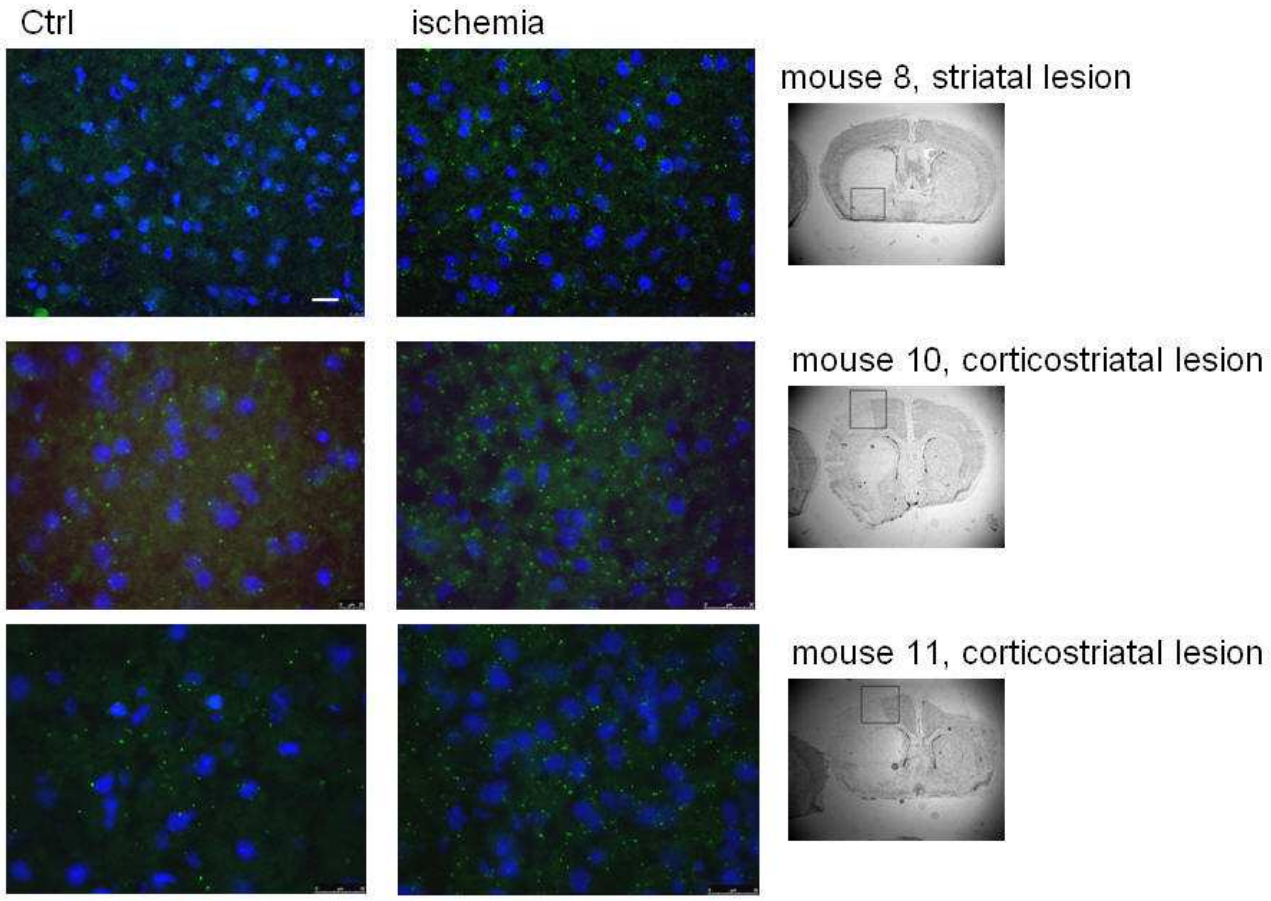

Figure 6) Cx36 immunolabelling is increased in the border of the ischemic hemisphere: representative immunofluorescence images comparing the punctate Cx36 labelling (appearing in green) between the control and ischemic hemisphere of three different mice brains. DAPI counterstaining appears blue. Scale bar=20µm

3.4. GFAP and Connexin43 expression in focal cerebral ischemia

In order to determine whether transient focal cerebral ischemia, rather than modulating Cx43 protein abundance, changed the distribution and/or the expression of Cx43 to the border of the ischemic lesion, we performed a double immunofluorescence labelling using specific antibodies against Cx43 and GFAP known to specifically stain the differentiated astrocytes.

To examine ischemia-induced alterations in Cx43 expression in astrocytes, consecutive slices from same mice brains used for cresyl violet staining and anti-cleaved caspase 3 studies were taken and concomitant anti-GFAP and anti-Cx43 immunofluorescence were performed.

It has been suggested that astrocytic gap junctions could be important for the regulation of neuronal apoptosis and the inflammatory response after an ischemic insult on brain tissue (Nakase, Sohl et al. 2004). Previous work suggested that reactive astrocytes (GFAP positive cells) are predominantly located in the spared parenchyma bordering the ischemic zone (Benakis, Bonny et al. 2010). Concurrently, Connexin43 protein abundance remains unchanged after global cerebral ischemia (Oguro, Jover et al. 2001).

We found that anti-GFAP immunofluorescence was decreased in the ischemic lesion, rather than augmented on the border (Figure 7).

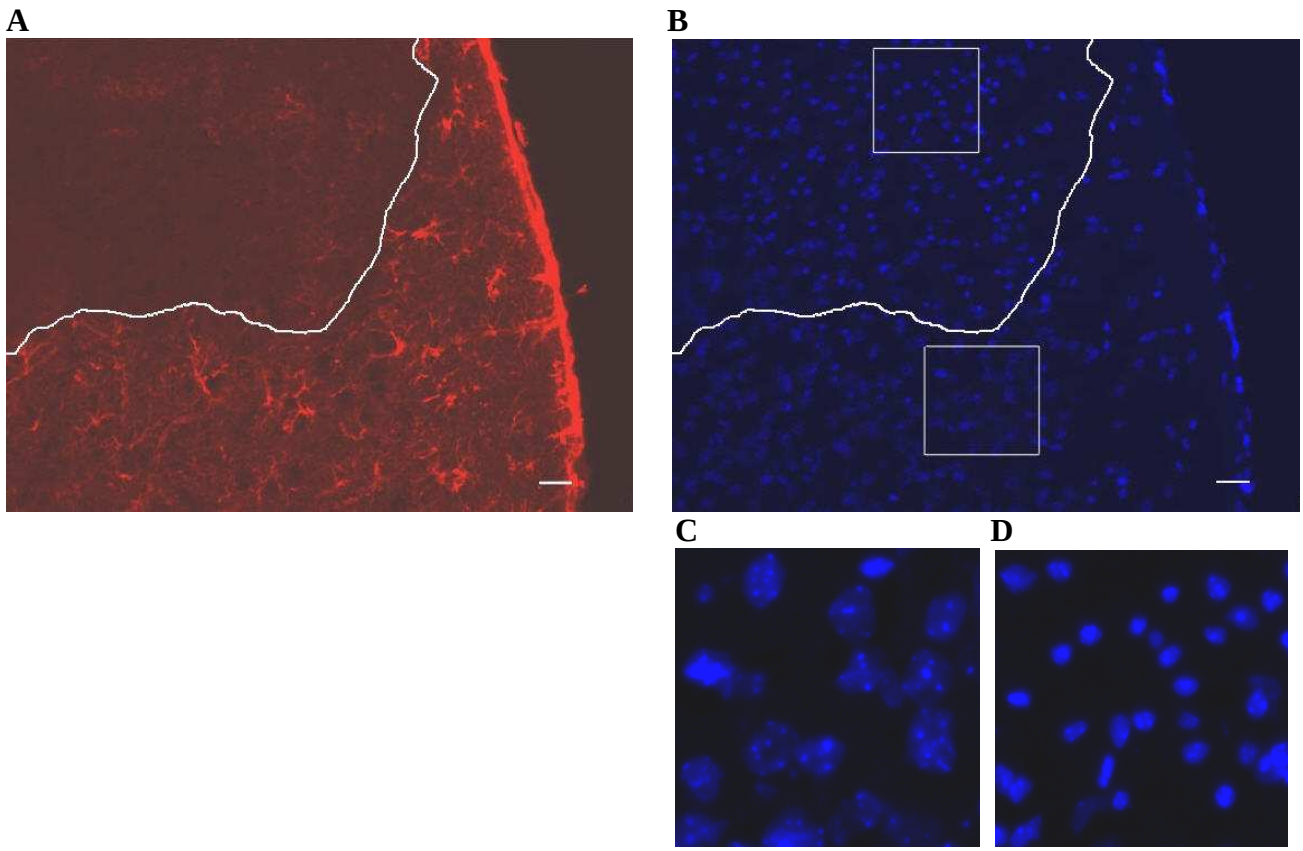

Figure 7) GFAP immunolabelling in the cortex: A: anti-GFAP labelling B: DAPI staining shows nuclear morphological differences between the ischemic lesion and the adjacent parenchyma. Squares indicate the magnifications C and D (C lower square, D upper square). Scale bar=50µm

Cx43 expression, assessed by immunofluorescence, was not modulated by a 30min transient focal cerebral ischemia. We didn't observe a difference between the ischemic and control brain tissue regarding Cx43 immunolabelling (Figure 8). Furthermore, there was no clear redistribution of Cx43 to the regions with more preponderant GFAP signal. The representative

pictures in Figure 8 show that the specific Cx43 labelling is more prevalent in regions without anti-GFAP labelling. Cx43 was either strongly localized as on mouse 8 tissue slices, or detected as a rather diffuse punctate as shown on mouse 30 tissue.

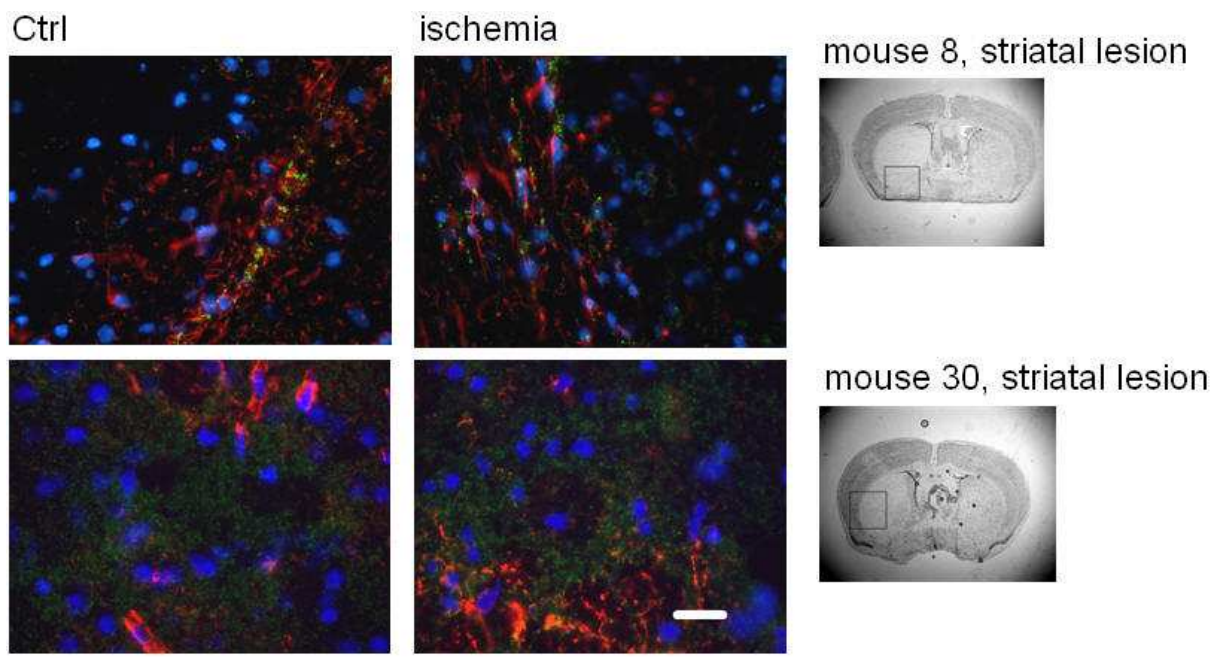

Figure 8) Concomitant GFAP and Cx43 immunolabelling:
Comparison between the ischemic and non-ischemic hemisphere. GFAP labelling appears in red, Cx43 labelling appears in green, DAPI counterstaining appears in blue. Scale bar=20µm

3.5. Transient focal cerebral ischemia induces no detectable modulation in Connexin36 protein abundance in ischemic tissue

To assess semi-quantitatively the effects of transient focal cerebral ischemia on Cx36 expression, we performed Western blot analysis. The ischemic lesion was isolated from adjacent normal tissue and samples of whole cell protein were subjected to gel electrophoresis and incubated with specific antibodies against Cx36. As a positive control, we also examined Cx36 expression in the insulin-secreting INS-1 beta-cells which together with neurons exclusively express Cx36 (Martin, Tawadros et al. 2003). This preliminary work showed a slightly more intense signal in some of the control tissue samples (8, 10, and 11) in the Western blot done without concomitant tubulin labelling to assure equivalent protein loading

in all pits (Figure 9A). The INS-1 protein sample showed an intense, specific signal for the antibody against Cx36.

In Figure 9B, although there was specific anti-Cx36 signal in the INS-1 protein sample at 36kDa, the signal in the brain protein samples is positive at 28kDa as indicated by the protein molecular weight marker. Also, we didn't observe a detectable difference in Cx36 expression between ischemic and control tissue. Anti-tubulin signal demonstrated that the amount of proteins loaded on the gel were similar between the samples.

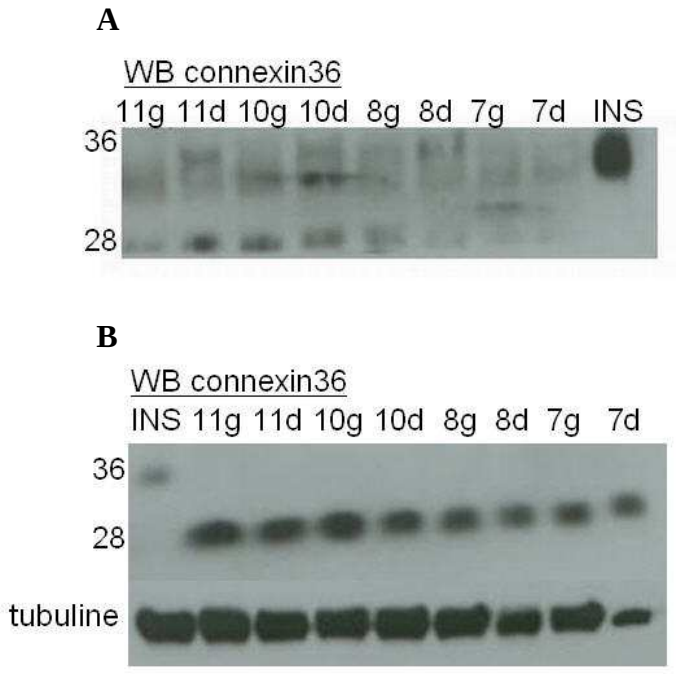

Figure 9) Western blot Connexin36:
Numbers 7, 8, 10, 11 indicate different mice brains, d: right hemisphere, control, g: left hemisphere, concentrated ischemic tissue. INS: beta cells used as positive control

To assess quantitatively effects of transient focal cerebral ischemia on Cx43 expression, we performed Western blot analysis. The ischemic lesion was isolated from adjacent normal tissue and samples of whole cell protein were subjected to gel electrophoresis and incubated with specific antibodies against Cx43. As a negative control, we also examined Cx43 expression in INS-1 beta-cells which do not express Cx43.

There was no difference in Cx43 expression seen between the ischemic and control tissue. Anti-tubulin signal demonstrated that the amount of proteins loaded on the gel were similar between the samples (Figure 10).

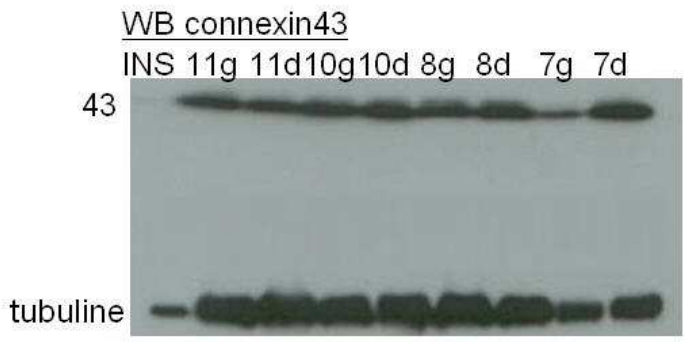

Figure 10) Western blot Connexin43:
Numbers 7, 8, 10, 11 indicate different mice brains, d: right hemisphere, control, g: left hemisphere, concentrated ischemic tissue. INS: beta cells used as negative control.

4. Discussion and Perspectives

The present work was undertaken to examine the regulation of the gap junction protein Connexin36 in neurons in a mouse model of middle cerebral artery occlusion-induced transient focal cerebral ischemia.

We observed a qualitative increase of Cx36 immunofluorescence signal in the penumbral region of the ischemic hemisphere compared to control tissue in 6 out of 19 different mice subjected to 30min of transient focal cerebral ischemia induced by MCAo.

This observation is in accordance with results from a previous study (Oguro, Jover et al. 2001) and compatible with a role for Cx36 in survival and/or apoptosis of penumbral neurons.

In contrast to the study mentioned above which used 20min of global ischemia induced by bilateral occlusion of the arteria carotis communis, the present work used the MCAo mouse model for transient focal cerebral ischemia, creating a localized ischemic lesion with a surrounding penumbral region. This model enables the study of the ischemic penumbra while

being closer to the pathophysiological reality of a human cerebrovascular accident (85% of all cerebrovascular accidents are of thrombo-embolic nature, 25% in the territory of arteria cerebri media). It is unclear why the increase in Cx36 expression in the border of the penumbral region was observed in only 6 out of 19 mice. One possibility could be variations in the MCAo model between the mice as anatomical variations of collaterals in brain perfusion lead to differences in residual cerebral blood flow during MCAo or variable post-surgical mouse activity/seizures leading to more or less oxygen demand of tissues. These explanations are in accordance with the observation that repeated immunofluorescence studies on different slices of the same mouse brain led to comparable results.

On the other hand, personal experience and handling from mouse to microscope are obviously of importance. Working as a student with no antecedent laboratory training, errors might have occurred during manipulations leading to misinterpretation of the results. Finally, the punctate Cx36 signal on the microscope is weak compared to a nuclear DAPI counterstaining and the visualisation of the fluorescent positive signals as well as the interpretation of the observable data is not easy for an untrained person.

The absence of a detectable modulation in Cx36 protein abundance in ischemic tissue assessed by Western blot analysis is in conflict with previous work (Oguro, Jover et al. 2001), who observed that 20min of global cerebral ischemia increased Cx36 protein abundance. An explanation for this could be the fact that, in contrast to the model of global cerebral ischemia, where a sample from any part of the brain can be used for Western blot analysis, we had to isolate the ischemic lesion tissue. Not only it is difficult to macroscopically determine the pathophysiological border of the lesion on cryosections of brain tissue, but also the isolation of the lesion with a surgical blade is not easily done. It is likely that we “diluted” the ischemic lesion tissue with the adjacent normal tissue in a way that the relatively small increase of Cx36 protein abundance in the lesion is negligible compared to the overall Cx36 protein abundance in the whole cell protein sample observed.

The main problem that we encountered during the Western blot analysis was related to the molecular weight of the Cx36 protein in brain tissue. Using INS-1E cells known to express Cx36 as a positive control, we observed a band at 36kDa in the control as expected, whereas the molecular size of the Cx36 immunodetected band in the brain extracts appeared at a size of 28kDa. It is possible that the specific band that we should observe at 36kDa was not detected in brain samples due to the lower expression levels of Cx36 in brain tissue (made of different types of cells which do not expressed Cx36) compared to INS-1E cells known to express high levels of Cx36 (Figure 9A). The use of brain samples from mice deleted for Cx36 (KO-Cx36), which are viable and lack behavioural or morphological abnormalities (Talhouk, Zeinieh et al. 2008) may be helpful to resolve this discrepancy.

The application of the MCAo transient focal cerebral ischemia model KO-Cx36mice would be a valuable model for the evaluation and comparison of the lesion volume between wild-type and transgenic mice. This may help to better understand the importance and role of Cx36 mediated intercellular communication in the destiny of penumbral neurons.

The Cx43 expressed in astrocytes has been shown to be involved in the pathology of neuronal disorders, including brain ischemia (Nakase, Sohl et al. 2004). As global cerebral ischemia did not alter Cx43 protein abundance (Oguro, Jover et al. 2001) and reactive astrocytes (GFAP positive cells) are predominantly located in the spared parenchyma bordering the ischemic zone (Benakis, Bonny et al. 2010), we studied whether transient focal cerebral ischemia, rather than modulating overall Cx43 protein abundance, changed the distribution of Cx43 in the border of the ischemic lesion, together with the GFAP positive cells. Even though it appeared difficult to assess the amount of anti-GFAP immunofluorescence signal on microscopic study, we found that the signal was diminished in the lesion, rather than increased around the lesion. Also, this was only possible to determine in brains with a corticostriatal lesion. Indeed, the corpus callosum, which surrounds the striatum and consequently a striatal ischemic lesion, is naturally rich in astrocytic GFAP (observed on the

control hemisphere). It was thus difficult to determine a modulation of the GFAP signal bordering the lesion.

We performed co-immunolocalization studies of GFAP and Cx43 to visualize a possible modulation of GFAP in association with a regulation of Cx43 labelling. We didn't find a global difference concerning Cx43 expression between ischemic and control tissue, which was also observed by Oguro et al. (Oguro, Jover et al. 2001). Furthermore, we didn't observe a modulation of Cx43 expression in GFAP-rich regions. In Figure 10 though, it seems that the specific Cx43 labelling is more prevalent in regions without GFAP labelling. It may be possible that the relatively weak Cx43 signal is masked by the much more intense GFAP signal on positions were both signals overlap.

Characterizing temporal, spatial and cellular differences in connexin expression is crucial to better understand the role of these different proteins in normal and pathophysiological conditions. The events taking place in an ischemic brain are complex and depend on different cell types and different types of intercellular communication. Both inflammation and hypoxia result in scenarios that add to the complexity of the issue (Talhouk, Zeinieh et al. 2008; Benakis, Bonny et al. 2010).

The role of connexins in the pathology of diseases in the central nervous system is still ambiguous and unravelling the function of gap junctions in the neural cell network, involving neurons, astrocytes and oligodendrocytes is of critical importance (Nakase and Naus 2004; Talhouk, Zeinieh et al. 2008).

A better understanding of the role of gap junctions may contribute to the development of new therapeutic approaches to treat brain diseases, including cerebral ischemia.

5. REFERENCES

Benakis, C., C. Bonny, et al. (2010). "JNK inhibition and inflammation after cerebral ischemia." Brain, behavior, and immunity **24**(5): 800-811.

Condorelli, D. F., N. Belluardo, et al. (2000). "Expression of Cx36 in mammalian neurons." Brain Res Brain Res Rev **32**(1): 72-85.

Condorelli, D. F., A. Trovato-Salinaro, et al. (2003). "Cellular expression of connexins in the rat brain: neuronal localization, effects of kainate-induced seizures and expression in apoptotic neuronal cells." Eur J Neurosci **18**(7): 1807-1827.

Goodenough, D. A. and D. L. Paul (2009). "Gap junctions." Cold Spring Harb Perspect Biol **1**(1): a002576.

Kumar, V. and S. L. Robbins (2007). Robbins basic pathology. Philadelphia, PA, Saunders/Elsevier.

Kunz, A., L. Park, et al. (2007). "Neurovascular protection by ischemic tolerance: role of nitric oxide and reactive oxygen species." The Journal of neuroscience : the official journal of the Society for Neuroscience **27**(27): 7083-7093.

Lin, J. H., H. Weigel, et al. (1998). "Gap-junction-mediated propagation and amplification of cell injury." Nature neuroscience **1**(6): 494-500.

Martin, D., T. Tawadros, et al. (2003). "Critical role of the transcriptional repressor neuron-restrictive silencer factor in the specific control of connexin36 in insulin-producing cell lines." J Biol Chem **278**(52): 53082-53089.

Mehta, S. L., N. Manhas, et al. (2007). "Molecular targets in cerebral ischemia for developing novel therapeutics." Brain research reviews **54**(1): 34-66.

Nakase, T., S. Fushiki, et al. (2003). "Neuroprotective role of astrocytic gap junctions in ischemic stroke." Cell Commun Adhes **10**(4-6): 413-417.

Nakase, T. and C. C. Naus (2004). "Gap junctions and neurological disorders of the central nervous system." Biochimica et Biophysica Acta **1662**(1-2): 149-158.

Nakase, T., G. Sohl, et al. (2004). "Increased apoptosis and inflammation after focal brain ischemia in mice lacking connexin43 in astrocytes." Am J Pathol **164**(6): 2067-2075.

Oguro, K., T. Jover, et al. (2001). "Global ischemia-induced increases in the gap junctional proteins connexin 32 (Cx32) and Cx36 in hippocampus and enhanced vulnerability of Cx32 knock-out mice." J Neurosci **21**(19): 7534-7542.

Ozog, M. A., R. Siushansian, et al. (2002). "Blocked gap junctional coupling increases glutamate-induced neurotoxicity in neuron-astrocyte co-cultures." Journal of neuropathology and experimental neurology **61**(2): 132-141.

Paciaroni, M., V. Caso, et al. (2009). "The concept of ischemic penumbra in acute stroke and therapeutic opportunities." European neurology **61**(6): 321-330.

Sohl, G., S. Maxeiner, et al. (2005). "Expression and functions of neuronal gap junctions." Nat Rev Neurosci **6**(3): 191-200.

Talhouk, R. S., M. P. Zeinieh, et al. (2008). "Gap junctional intercellular communication in hypoxia-ischemia-induced neuronal injury." Progress in neurobiology **84**(1): 57-76.

Tuttolomondo, A., D. Di Raimondo, et al. (2008). "Inflammatory cytokines in acute ischemic stroke." Current pharmaceutical design **14**(33): 3574-3589.

Waxman, S. G. (2010). Clinical neuroanatomy. New York, McGraw-Hill Medical.

Xia, W., J. Han, et al. (2010). "Inflammation in ischaemic brain injury: current advances and future perspectives." Clinical and experimental pharmacology & physiology **37**(2): 253-258.